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Ability of *Bifidobacterium breve* To Grow on Different Types of Milk: Exploring the Metabolism of Milk through Genome Analysis▽

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We have investigated the occurrence of bifidobacteria in human milk samples, and we provide evidence regarding the predominance of members of the *Bifidobacterium breve* species in this environment. Moreover, evaluation of the growth capabilities and transcriptomic analyses of one representative isolate of this species, i.e., *B. breve* 4L, on different milk types were performed.

The human intestine is sterile at birth but is then rapidly colonized by bacteria, ultimately leading to the development of a complex intestinal microbiota (4). Bifidobacteria are among the first bacterial colonizers of the intestine of neonates and are believed to represent the largest fraction of the infant microbiota (6, 27–30). Recently, bifidobacteria have been isolated from human milk, suggesting vertical mother-to-child transmission from the maternal gut to that of breast-fed infants (4, 14). However, an alternative hypothesis is that bifidobacteria are introduced into human milk through newborn-mother contacts, resulting in breast colonization by bifidobacteria that are present in the infant oral cavity during suckling, and that infant colonization occurs during birth from specific components of the mother's fecal/vaginal microbiota.

Human milk represents a continuous supply of nutrients to the neonate and also stimulates growth of specific bacterial groups/species in the infant intestine (23). Only a few species of the *Bifidobacterium* genus, such as *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*, are considered typical inhabitants of the infant intestine (13, 25). From an ecological context, (components of) the gut microbiota are considered to influence the health status of their host and, in particular, bifidobacteria have been claimed to positively impact the development and maintenance of a balanced immune system as well as to aid in the nutrition activities of intestinal cells (1, 7).

Recently, the genome sequences of various common infant bifidobacterial species, such as *Bifidobacterium longum* subsp. *infantis* ATCC 15697 and *Bifidobacterium bifidum* PRL2010 (26), have become publicly available (22). *In silico* as well as proteomic analyses revealed the existence of a large arsenal of genes encoding enzymes, such as fucosidases, sialidases, β-galactosidases, and *N*-acetyl-β-hexosaminidase, predicted to be involved in the metabolism of host glycans, like human milk oligosaccharides (HMOs) and mucin (22). However, the molecular mechanisms underlying the promotion of human milk to specific development of the intestinal bifidobacterial community is still poorly understood. In the present study we surveyed the bifidobacterial composition of the human milk microbiota and analyzed the molecular response of a milk-derived representative, i.e., *Bifidobacterium breve* 4L, during cultivation in human, cow, and formula milk, as well as in plant-derived milks.

Isolation and identification of bifidobacteria from human milk samples. The bifidobacterial population of the six collected human milk samples was assayed using mupirocin-based medium (BSM), which has previously been described to be selective for bifidobacteria (24, 27). Bifidobacterial cultures were incubated in an anaerobic atmosphere (2.99% H_2, 17.01% CO_2, and 80% N_2) in a chamber (Concept 400; Ruskin) at 37°C for 72 h. All 25 colonies that developed on BSM plates were subjected to DNA isolation by means of rapid mechanical cell lysis, as described previously (31). PCR was used to amplify the 16S sequences and intergenic transcribed sequences (ITSs) of these *Bifidobacterium* isolates by using previously described primers (27). To analyze the biodiversity of these cultivatable bifidobacteria, all isolated colonies were analyzed by sequencing of this section of the rRNA operon. Each 16S rRNA gene-ITS amplicon thus generated from individual colonies originating from milk samples was sequenced and was then subjected to a BLAST search against the GenBank database. All 25 sequences thus obtained showed more than 98% sequence identity to their nearest database entries, and thus no new bifidobacterial species were identified. Based on these BLAST results, all se-

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TABLE 1. *Bifidobacterium* isolates from human milk

Species	Code	Origin
B. breve	1L	Milk from subject 1
B. breve	2L	Milk from subject 5
B. breve	6L	Milk from subject 2
B. breve	4L	Milk from subject 4
B. breve	7L	Milk from subject 6
B. breve	9L	Milk from subject 2
B. breve	11L	Milk from subject 3
B. breve	12L	Milk from subject 3
B. breve	13L	Milk from subject 3
B. breve	14L	Milk from subject 3
B. breve	16L	Milk from subject 3
B. breve	17L	Milk from subject 3
B. longum subsp. *longum/infantis*	18L	Milk from subject 1
B. longum subsp. *longum/infantis*	19L	Milk from subject 2
B. longum subsp. *longum/infantis*	20L	Milk from subject 3
B. longum subsp. *longum/infantis*	21L	Milk from subject 4
B. adolescentis	22L	Milk from subject 3
B. adolescentis	26L	Milk from subject 6
B. longum subsp. *longum/infantis*	27L	Milk from subject 5
B. longum subsp. *longum/infantis*	28L	Milk from subject 6
B. adolescentis	29L	Milk from subject 5
B. breve	30L	Milk from subject 1
B. breve	31L	Milk from subject 6
B. longum subsp. *longum/infantis*	32L	Milk from subject 1
B. longum subsp. *longum/infantis*	33L	Milk from subject 5

quences were assigned to three phylogenetic taxa, representing *B. breve*, *Bifidobacterium longum* subsp. *longum/infantis*, and *Bifidobacterium adolescentis* (Table 1). The phylogenetic relationships between the 25 isolated bifidobacterial strains were analyzed using a 16S-rRNA gene-based tree, which clearly demonstrated separation into three distinct phylogenetic clusters and which corresponded to the bifidobacterial species identified by BLAST analysis (Fig. 1). Phylogenetic analysis showed that the majority of the investigated strains (56% of the total isolates) belonged to *B. breve*. As described previously, the high degree of variability of the ITSs at an intraspecific level in bifidobacteria renders this molecular marker suitable for the specific molecular characterization of bifidobacterial strains (28). When we compared the 16S rRNA gene-ITSs from all bifidobacteria isolated from human milk samples, we identified a level of similarity below 8%, thus suggesting that each of the isolated strains is genetically distinct (data not shown).

Evaluation of growth capabilities of different human milk isolates. All strains from each bifidobacterial species isolated from human milk, except for *B. adolescentis*, were assayed for their growth abilities on different milk-based substrates, i.e., human milk, bovine milk, formula milk, soybean-based milk, and rice-based milk. Although the isolation attempts led to the identification of three strains belonging to the *B. adolescentis* taxon, we decided not to include such strains in further experiments, due to the fact that members of this bifidobacterial species have previously been associated with infants suffering from allergic diseases (8).

Cell growth curves on these substrates of all these bifidobacterial strains were monitored for 48 h by a plate reader (Biotek, Winooski, VT), which was set as described previously (26, 32). About 10^9 CFU/ml for each bifidobacterial strain were inoculated in basic medium (20) supplemented with 0.5% (wt/vol) of a particular milk (i.e., bovine milk, reconstituted formula milk, human milk, soybean-based milk, and rice-based milk). We also tested growth on higher concentrations of milk in the growth medium without noticing differences (data not shown). The various milk types were pasteurized (80°C for 20 min) and kept at 4°C until use. Notably, a large variability of growth on milk-based media was evident among both the *B. breve* as well as *B. longum* strains, even if growth behavior was comparable for all the analyzed milk types (Fig. 2). Furthermore, mid-exponential phase was reached at similar times for the various milk types utilized. Notably, *B. breve* 4L showed the best growth ability on milk-based media (Fig. 2), thus prompting us to further investigate the behavior of this strain in order to obtain a better understanding of the molecular adaptation of this strain to utilize such milk products.

The milk sources used, i.e., bovine, reconstituted formula, human, soybean based, and rice based, displayed very different gross chemical compositions, which may explain the different growth performances shown by the strains analyzed. In fact, whereas mammalian-derived milks exhibit a high protein content (from 1.1% in human milk to 3.2% in cow milk), such as caseins and whey proteins, plant-derived milks do not contain such a high protein level. Furthermore, the carbon sources identified in these two different milk types are very different in terms of complexity and chemical composition, i.e., lactose in mammalian milk (2% in cow milk and 7% in human milk) versus sucrose and fructooligosaccharides in plant-derived milks.

Transcriptional profiling of *B. breve* 4L cells grown on different milk substrates and adaptation to the infant gut. Characteristics contributing to the ecological fitness in the infant gut, such as utilization of milk, should be discernible in *B. breve* 4L. To determine if *B. breve* 4L functionally and distinctly responds to different types of milks, we performed transcriptional profiling studies using CombiMatrix arrays (CombiMatrix, Mukilteo, WA) based on the genome sequence of *B. breve* DSM20213 (NCBI source NZ_ACCG00000000). Oligos were synthesized in 18 replicates on a 2x40K CombiMatrix array. Replicates were distributed on the chip at random, nonadjacent positions. A set of 29 negative-control probes designed on phage and plant sequences was also included on the chip in 60 replicates at randomly distributed positions. Three hybridization assays were carried out for each condition analyzed (experimental replicates). All experimental replicates showed a high correlation (>0.98).

To identify the potential transcriptional signatures of different types of milk, the transcriptome profiles recorded upon growth on the various milk substrates were compared to the transcriptome obtained after growth on MRS plus lactose. RNA samples for each culture condition were prepared from cells grown to mid-exponential phase.

Total RNA was isolated with the macaloid acid method (37) and treated with DNase (Roche, United Kingdom). Briefly, cell pellets were resuspended in 1 ml of QIAzol (Qiagen, United Kingdom) and placed in a tube containing 0.8 g of glass beads (diameter, 106 μm; Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer at 4°C for 2 min (maximum setting). The mixture was then centrifuged at 12,000 rpm for 15 min, and the RNA-containing upper phase was recovered. Each RNA sample was further purified by

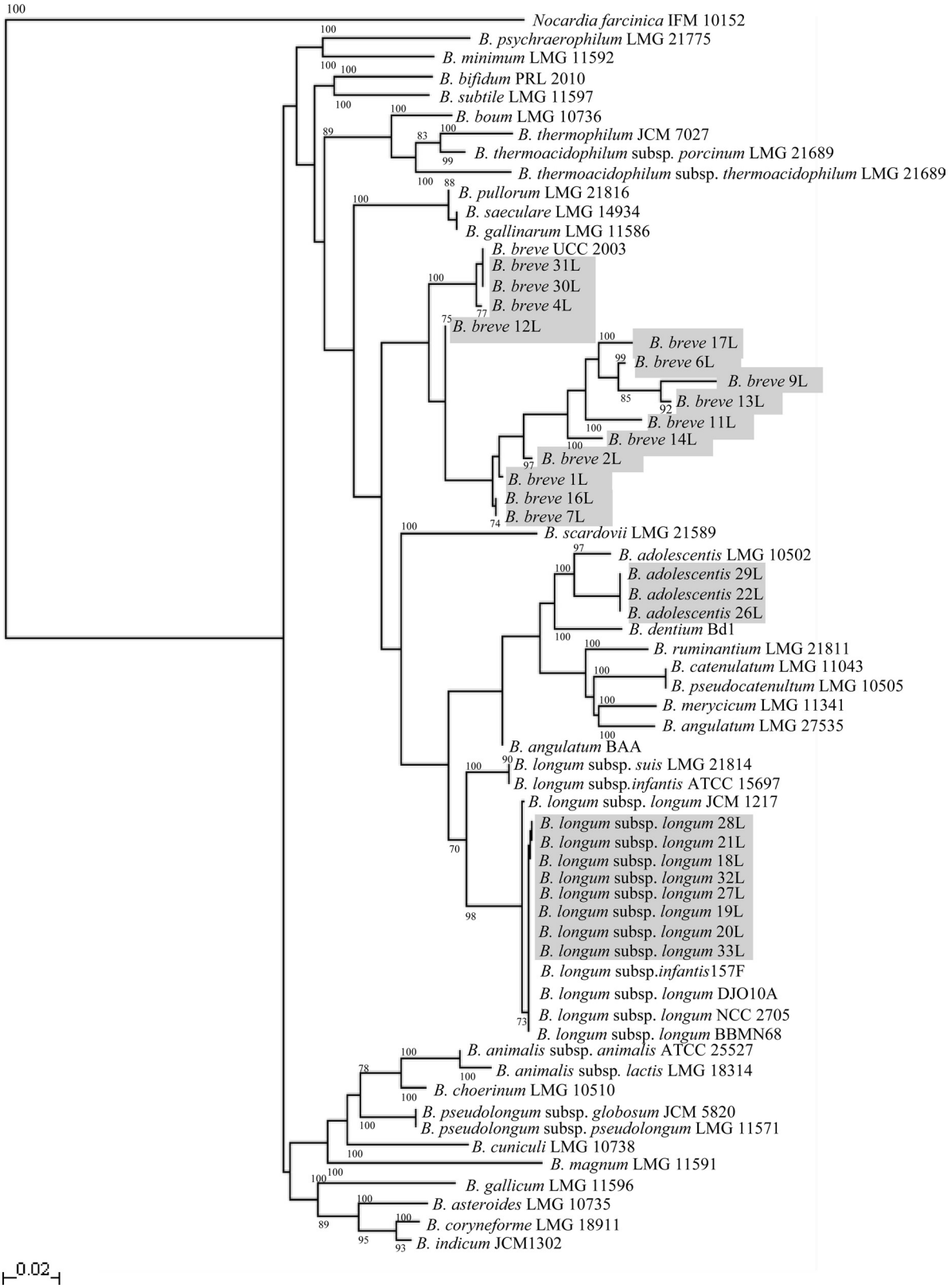

FIG. 1. Phylogenetic tree based on the 16S rRNA genes of human milk bifidobacterial isolates. Bootstrap values are indicated.

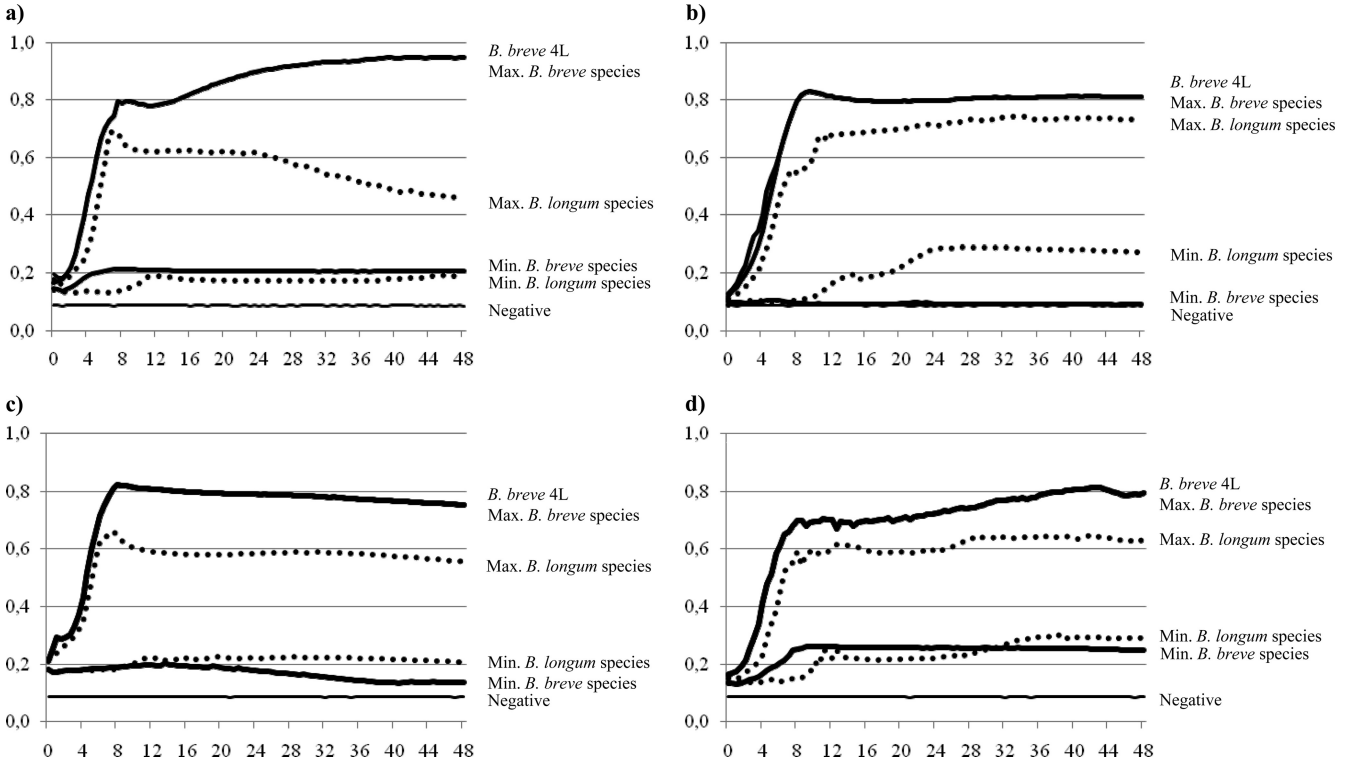

FIG. 2. Milk-metabolizing capabilities of human milk bifidobacterial isolates. Each panel shows the growth curves (optical density [OD] versus time [in h]) of human milk bifidobacterial isolates on human milk (a), bovine milk/formula milk (b), soybean-based milk (c), and rice-based milk (d). The curves representing the minimal and the maximal growth detected for each species are indicated. "Negative" represents the OD values detected in the basal medium without inocula.

phenol extraction and ethanol precipitation according to a previously described method (21).

Transcriptome analysis showed that a total of 623 genes were differentially transcribed upon growth of *B. breve* 4L cells on one or more of the test milk-based media compared to MRS plus lactose. A gene was considered to be differentially expressed between a test condition and a control when an expression ratio of >2 or <0.2 relative to the result for the control was obtained with a corresponding P value that was <0.001. As indicated by the cluster analysis data reported in Fig. 3a (derived from a representative subset of culture medium-modulated genes), distinct transcriptional profiles were associated with the different milk types under investigation. This was further corroborated by a global comparison of the transcription profiles associated with the six different growth media (Fig. 3b), which revealed a close relatedness between the animal milks, whose transcriptional response profiles markedly differed from those associated with the plant-based milks and the reference (MRS plus lactose).

As shown by the Venn diagram in Fig. 3c, increased transcription of 134 genes relative to the reference (MRS plus lactose) transcriptome was observed upon growth on either of the three mammalian milk types (i.e., human or bovine milk or formula milk), while the transcription levels of 163 genes selectively increased upon cultivation on plant-derived milks (i.e., soybean-based milk or rice-based milk).

Functional classification of milk source-upregulated genes indicated that most of them code for proteins (enzymes and transporters) involved in amino acid and carbohydrate metabolism (Fig. 4).

Genes preferentially expressed upon growth on mammalian-derived milks. Despite the abundance of carbohydrates in animal-derived milks, relatively few genes involved in carbohydrate metabolism and transport were found to be upregulated ($\geq$2.0-fold versus the lactose reference condition) during growth on human, cow, or formula milk (Table 2 and Fig. 4). These included a putative β-galactosidase (BIFBRE_04539), a galactoside symporter (BIFBRE_04540), and several ABC transporter systems and phosphotransferase system (PTS) components that are predicted to be directly or indirectly involved in sugar uptake (Table 2). This finding might be explained by the fact that animal milks contain high levels of lactose (~5% of the total content), which is a preferred carbon source that can suppress expression of genes involved in alternative carbohydrate metabolic pathways.

Among the genes whose expression was significantly upregulated upon cultivation of *B. breve* 4L on human, bovine, or formula milk were those encoding the galactose-1-phosphate-uridyltransferase (GalT) and the galactokinase (GalE) enzymes. These genes are part of the *lnpABCD* operon (17), which is responsible for the utilization of lacto-*N*-biose (LNB; Galβ1-3GlcNac). This carbohydrate forms the core structure of HMOs, which have been identified in human milk as important constituents that selectively stimulate the growth of bifidobacteria in the infant intestine (2, 35) and represent the third most abundant component of human milk, after lactose

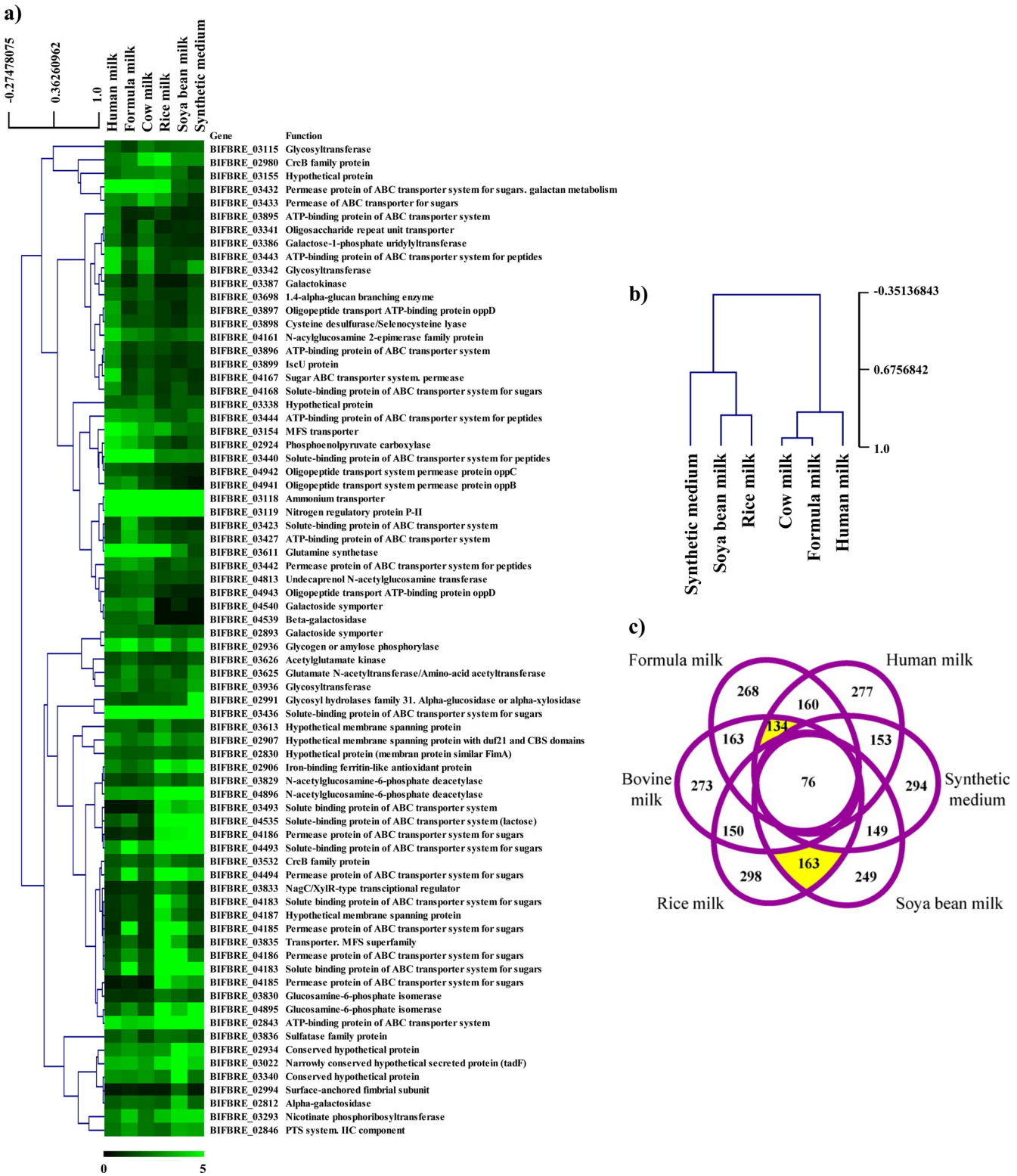

FIG. 3. Identification of *B. breve* 4L transcribed genes by DNA microarray analysis. (a) Change in expression of selected genes upon cultivation of 4L cells in different milk-based substrates (human milk, bovine milk, formula milk, rice-based milk, and soybean-based milk) and on MRS with glucose as the unique carbon source, compared to growth on MRS plus lactose. Each row represents a separate transcript, and each column represents a separate sample. Green indicates increased transcription levels, whereas black indicates decreased transcription levels compared to the reference samples (lactose-grown samples). The level of transcription is provided at the bottom of the figure. (b) *B. breve* 4L transcriptomics clustering analysis was performed for the six substrates. (c) Venn diagram of the upregulated genes when *B. breve* 4L cells are cultivated on human milk, bovine milk, formula milk, rice-based milk, soybean-based milk, or on MRS with glucose, compared to MRS plus lactose as unique carbon source. Circle sizes are proportional to the number of upregulated genes contained in each set.

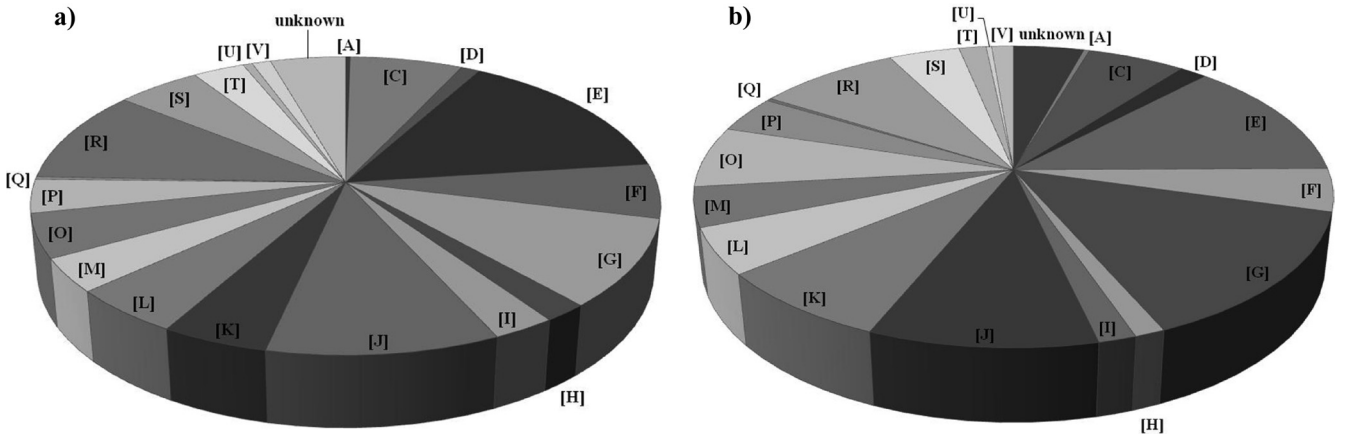

FIG. 4. Classification of the *B. breve* 4L genes differentially expressed in the presence of different milk substrates, according to COG functional categories. (a) COG categories of 4L genes upregulated in the presence of mammal-derived milk. (b) COG categories of 4L genes overexpressed in the presence of plant-derived milk. Each COG family is identified by a one-letter abbreviation: A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication and repair; M, cell wall/membrane/envelop biogenesis; N, cell motility; O, posttranslational modification, protein turnover, chaperone functions; P, inorganic ion transport and metabolism; Q, secondary structure; T, signal transduction; U, intracellular trafficking and secretion; Y, nuclear structure; V, defense mechanisms; Z, cytoskeleton; R, general functional prediction only; S, function unknown.

and lipids (11, 34). The observed upregulation of this gene cluster is consistent with previous findings reported for *B. longum* cultivated on breast milk (5).

Notably, genes involved in glutamate metabolism were found to be upregulated during growth of *B. breve* on human, cow, and formula milk compared to MRS plus lactose. These included an ABC transporter specific for glutamate (BIFBRE_04715 and BIFBRE_04716), whose activity may be linked to nitrogen metabolism through the condensation of glutamate and ammonia to form glutamine (5). Other gene products linked to glutamine synthesis, such as glutamine synthetase itself (BIFBRE_03611) and *N*-acetylglutamate transferase (BIFBRE_03625), were also significantly upregulated in *B. breve* cells cultured on formula milk compared to MRS plus lactose.

Also worth noting is a genetic locus comprised of five genes (BIFBRE_03895 to BIFBRE_03899), coding for an ABC transporter, an oligopeptide binding protein, a cysteine desulfatase, and a sulfur cluster assembly complex, that was selectively upregulated upon growth on human milk but not on cow or formula milk. This finding may reflect the presence of higher levels of sulfur-containing amino acids (e.g., cysteine) in human milk than in either bovine or formula milk (3).

Genes preferentially expressed upon cultivation of *B. breve* 4L on plant-derived milks. There were 163 genes significantly upregulated upon cultivation of *B. breve* 4L on the two tested plant-derived milks (soybean and rice) compared to animal/human or formula-based milk (Fig. 3c). Among these differentially expressed genes there were various open reading frames annotated as gene products involved in carbohydrate utilization, including an ABC transporter system (BIFBRE_04186 to BIFBRE_04183) and an α-glucosidase (BIFBRE_02991). Furthermore, a genetic locus encompassing three genes predicted to encode a glucosamine-6-phosphate isomerase, a glucosamine-6-phosphate deacetylase, and a NagC transcriptional regulator was highly upregulated in both soybean-based milk and rice-based milk compared to animal-derived milks.

Of note, two genes (BIFBRE_02994 and BIFBRE_02830) coding for proteins similar to the type II fimbrial major subunit (FimA) of *Actinomyces naeslundii* were upregulated upon cultivation of *B. breve* 4L on soybean-based milk and rice-based milk. In closely related microorganisms, such as *Corynebacterium diptheriae* and *A. naeslundii*, fimbria are crucial for bacterial adherence to specific host cells, including epithelial cells, erythrocytes, and polymorphonuclear leukocytes (12, 16), as well as for binding to other bacteria (15, 36), a scenario which also applies to human gut commensals, such as lactobacilli (9, 33).

Genes upregulated during growth in both mammalian- and plant-derived milks. The transcription of 76 genes was shown to be significantly upregulated upon cultivation of *B. breve* 4L on animal and plant milks compared to MRS plus lactose as the only carbon source (Fig. 3c). Among these genes were those involved in the synthesis of amino acids, such as chorismate mutase (BIFBRE_03188) and chrorismate synthase (BIFBRE_03889), which are part of the shikimate pathways responsible for the metabolism of aromatic amino acids, such as phenylalanine and tyrosine, and hystidinal dehydrogenase (BIFBRE_04783), which is involved in histidine metabolism. In addition, expression levels of BIFBRE_03118 and BIFBRE_03119, which encode, respectively, an ammonium transporter and a nitrogen regulatory protein, were found to be more than 6-fold higher in milk-cultured bacteria than in bacteria grown on the reference synthetic medium. This finding, together with the upregulation of genes involved in amino acid metabolism, indicates that nitrogen assimilation in *B. breve* 4L mainly proceeds through peptide hydrolysis. Indeed, increased levels of transcripts coding for an endopeptidase (BIFBRE_02980), a predicted peptidase (BIFBRE_03114), and a putative aminopeptidase (BIFBRE_04898) were also detected in *B. breve* 4L cells cultured on animal or plant milk. Other upregulated genes shared by both milk types included a

TABLE 2. Selected transcripts upregulated in cells cultivated in different milk-based samples compared to *B. breve* 4L grown on MRS plus lactose

Gene	Function	Fold change above control[a] (*P* value) for each milk source					
		Human	Formula	Cow	Rice based	Soybean based	Synthetic medium
BIFBRE_02893	Galactoside symporter	2.15 (2.60E−05)	2.25 (3.78E−05)	1.77 (2.20E−04)	1.99 (5.96E−05)	1.73 (5.32E−03)	2.01 (5.32E−03)
BIFBRE_02991	α-Glucosidase	1.80 (6.72E−01)	1.46 (9.88E−01)	1.76 (3.32E−03)	1.82 (1.98E−01)	2.03 (6.54E−02)	5.62 (6.54E−02)
BIFBRE_04186	Permease protein of ABC transporter system	0.81 (1.12E−04)	1.12 (2.05E−04)	0.78 (4.73E−01)	4.72 (6.66E−05)	4.84 (3.48E−04)	5.49 (3.48E−04)
BIFBRE_04185	Permease protein of ABC transporter system	0.50 (9.36E−02)	0.79 (3.34E−01)	0.44 (4.73E−01)	4.96 (1.96E−06)	3.69 (2.23E−06)	3.07 (2.23E−06)
BIFBRE_04183	Solute binding protein of ABC transporter system	1.10 (3.97E−01)	1.57 (2.59E−01)	1.04 (1.86E−02)	6.23 (3.51E−05)	3.30 (2.50E−03)	1.34 (2.50E−03)
BIFBRE_02906	Iron binding ferritin-like antioxidant protein	2.75 (6.98E−01)	2.07 (7.99E−02)	2.54 (3.32E−03)	6.44 (3.65E−05)	4.24 (6.58E−04)	6.01 (6.58E−04)
BIFBRE_02907	Hypothetical membrane-spanning protein	3.12 (6.98E−01)	2.20 (7.99E−02)	2.61 (3.32E−03)	3.77 (3.65E−05)	2.43 (6.58E−04)	2.83 (6.58E−04)
BIFBRE_02924	Phosphoenolpyruvate carboxylase	4.57 (5.71E−01)	3.84 (4.21E−01)	2.85 (4.73E−01)	1.78 (2.14E−01)	1.15 (4.88E−05)	1.87 (4.88E−05)
BIFBRE_02934	Conserved hypothetical protein	2.72 (5.37E−01)	3.12 (7.22E−01)	2.83 (3.32E−03)	3.03 (3.82E−01)	6.86 (6.31E−03)	4.47 (6.31E−03)
BIFBRE_02936	Glycogen or amylose phosphorylase	4.37 (1.09E−03)	5.41 (5.52E−04)	3.42 (3.32E−03)	4.55 (5.03E−04)	2.67 (2.30E−05)	4.19 (2.30E−05)
BIFBRE_02980	CrcB family protein	2.35 (9.53E−03)	2.68 (6.61E−01)	4.58 (3.32E−03)	4.99 (2.59E−01)	2.88 (6.69E−01)	2.84 (6.69E−01)
BIFBRE_02994	FimA	0.55 (6.96E−01)	0.71 (1.65E−01)	0.57 (1.86E−02)	0.59 (1.84E−01)	2.08 (4.20E−01)	0.60 (4.20E−01)
BIFBRE_03022	TadE/TadF protein	3.57 (2.66E−03)	3.72 (2.33E−02)	3.14 (3.32E−03)	4.53 (1.24E−03)	6.23 (1.69E−02)	4.11 (1.69E−02)
BIFBRE_03022	TadE/TadF protein	3.59 (3.37E−04)	3.78 (1.34E−03)	3.26 (6.61E−01)	5.03 (3.32E−03)	5.50 (2.16E−04)	3.78 (2.17E−06)
BIFBRE_03114	Dipeptidase A	2.55 (5.81E−02)	3.02 (3.37E−04)	3.23 (1.85E−02)	2.73 (2.09E−03)	2.62 (1.02E−04)	2.76 (3.00E−03)
BIFBRE_03115	Glycosyltransferase	2.04 (3.75E−03)	1.30 (9.75E−03)	2.55 (3.32E−03)	2.00 (5.89E−04)	2.15 (2.99E−02)	2.20 (2.99E−02)
BIFBRE_03118	Ammonium transporter	10.16 (2.29E−03)	24.16 (4.40E−03)	21.60 (4.52E−04)	9.05 (4.73E−02)	6.01 (1.87E−02)	7.94 (1.87E−02)
BIFBRE_03119	Nitrogen regulatory protein P-II	15.88 (2.17E−06)	53.85 (5.91E−07)	43.52 (4.73E−01)	21.35 (2.22E−06)	12.60 (8.52E−06)	9.02 (8.52E−06)
BIFBRE_03154	MFS transporter	5.19 (5.33E−04)	4.55 (1.85E−02)	3.24 (3.32E−03)	3.70 (5.81E−02)	2.27 (2.65E−01)	2.04 (2.65E−01)
BIFBRE_03155	Hypothetical protein	2.06 (5.33E−04)	2.66 (1.85E−02)	2.65 (3.32E−03)	3.07 (5.81E−02)	2.32 (2.65E−01)	1.19 (2.65E−01)
BIFBRE_03188	Chorismate mutase	2.97 (2.16E−04)	3.61 (8.29E−05)	3.41 (1.02E−04)	2.22 (1.34E−03)	2.80 (3.37E−04)	2.24 (1.31E−03)
BIFBRE_03293	Nicotinate phosphoribosyltransferase	2.52 (8.68E−03)	4.00 (4.07E−02)	2.35 (4.73E−01)	3.76 (1.70E−01)	4.49 (5.17E−01)	4.49 (5.17E−01)
BIFBRE_03432	Permease protein	5.73 (2.09E−03)	4.99 (3.00E−03)	10.15 (4.73E−01)	4.90 (1.31E−03)	2.03 (1.16E−01)	1.81 (1.16E−01)
BIFBRE_03338	Hypothetical protein	2.04 (1.55E−02)	2.01 (7.84E−01)	2.58 (3.32E−03)	1.24 (5.58E−03)	1.84 (2.39E−01)	1.79 (2.39E−01)
BIFBRE_03340	Conserved hypothetical protein	2.92 (5.13E−03)	2.77 (5.87E−03)	3.05 (3.32E−03)	2.50 (2.96E−01)	6.83 (1.23E−02)	2.29 (1.23E−02)
BIFBRE_03341	Oligosaccharide repeat unit transporter	2.61 (2.29E−03)	0.69 (3.16E−03)	2.50 (4.73E−01)	0.80 (5.08E−03)	0.98 (7.33E−05)	1.05 (7.33E−05)
BIFBRE_03342	Glycosyltransferase	4.44 (2.91E−04)	1.34 (5.00E−02)	3.54 (3.32E−03)	1.36 (2.03E−01)	1.66 (9.24E−01)	3.40 (9.24E−01)
BIFBRE_03386	Galactose-1-phosphate uridylyltransferase	2.36 (1.51E−02)	0.93 (7.13E−01)	2.30 (3.32E−03)	1.22 (1.96E−03)	0.98 (9.41E−03)	0.90 (9.41E−03)
BIFBRE_03387	Galactokinase	2.05 (1.53E−03)	0.72 (7.37E−01)	2.03 (3.32E−03)	0.57 (3.29E−01)	0.56 (9.20E−01)	1.37 (9.20E−01)
BIFBRE_03423	Solute binding protein of ABC transporter system	1.53 (1.36E−04)	4.11 (2.76E−03)	1.97 (1.86E−02)	1.29 (7.20E−04)	1.03 (1.57E−03)	0.80 (1.57E−03)
BIFBRE_03427	ATP binding protein of ABC transporter system	1.82 (5.48E−02)	3.83 (3.59E−03)	2.39 (3.10E+04)	1.82 (5.55E−01)	1.41 (9.06E−01)	1.63 (9.06E−01)
BIFBRE_03433	Permease of ABC transporter for sugars	2.83 (4.14E−04)	2.58 (5.16E−04)	4.16 (4.73E−01)	3.23 (4.81E−04)	1.69 (2.58E−04)	0.93 (2.58E−04)
BIFBRE_03436	Solute binding protein of ABC transporter system	7.68 (4.14E−04)	8.28 (5.16E−04)	8.99 (1.86E−02)	10.36 (4.81E−04)	7.80 (2.58E−04)	12.34 (2.58E−04)
BIFBRE_03440	Solute binding protein of ABC transporter system for peptides	9.81 (4.14E−04)	6.78 (5.16E−04)	5.94 (1.86E−02)	2.65 (4.81E−04)	2.74 (2.58E−04)	2.59 (2.58E−04)
BIFBRE_03442	Permease protein of ABC transporter system for peptides	2.93 (4.14E−04)	3.41 (5.16E−04)	2.94 (4.73E−01)	1.49 (4.81E−04)	2.07 (2.58E−04)	1.74 (2.58E−04)
BIFBRE_03443	ATP binding protein of ABC transporter system for peptides	4.45 (4.14E−04)	1.92 (5.16E−04)	3.76 (1.36E−07)	1.40 (4.81E−04)	1.27 (2.58E−04)	1.40 (2.58E−04)
BIFBRE_03444	ATP binding protein of ABC transporter system for peptides	3.46 (4.14E−04)	3.07 (5.16E−04)	2.93 (9.09E−04)	2.00 (4.81E−04)	1.76 (2.58E−04)	2.58 (2.58E−04)
BIFBRE_03493	Solute binding protein of ABC transporter system	0.47 (6.10E−01)	0.46 (1.00E+00)	0.70 (1.86E−02)	4.62 (1.22E−01)	3.59 (5.85E−01)	4.09 (5.85E−01)
BIFBRE_03532	CrcB family protein	1.67 (8.33E−03)	2.19 (1.45E−02)	1.58 (3.32E−03)	3.07 (6.82E−01)	2.14 (1.39E−02)	1.80 (1.39E−02)
BIFBRE_03611	Glutamine synthetase	9.05 (1.31E−01)	22.81 (8.83E−01)	12.46 (3.32E−03)	5.42 (2.58E−02)	3.04 (6.67E−01)	1.47 (6.67E−01)
BIFBRE_03613	Hypothetical membrane-spanning protein	2.12 (9.95E−06)	2.16 (2.29E−06)	1.58 (3.32E−03)	3.13 (3.97E−05)	1.67 (6.75E−04)	1.75 (6.75E−04)
BIFBRE_03625	Glutamate N-acetyltransferase	1.94 (1.74E−02)	3.01 (3.26E−03)	1.76 (3.32E−03)	2.29 (9.68E−03)	1.53 (2.89E−01)	3.22 (2.89E−01)
BIFBRE_03626	Acetylglutamate kinase	1.53 (1.74E−02)	2.14 (3.26E−03)	1.29 (2.07E+04)	1.17 (9.68E−03)	1.28 (2.89E−01)	1.91 (2.89E−01)
BIFBRE_03698	1,4-α-Glucan branching enzyme	2.50 (2.76E−02)	1.57 (7.64E−04)	2.03 (3.90E+03)	1.06 (1.81E−04)	1.07 (5.03E−02)	1.70 (5.03E−02)
BIFBRE_03829	*N*-Acetylglucosamine-6-phosphate deacetylase	1.51 (2.93E−04)	1.24 (1.86E−04)	1.64 (3.32E−03)	2.58 (2.51E−01)	1.78 (4.97E−03)	2.41 (4.97E−03)
BIFBRE_03830	Glucosamine-6-phosphate isomerase	1.04 (2.93E−04)	0.98 (1.86E−04)	1.11 (3.32E−03)	2.46 (2.51E−01)	2.03 (4.97E−03)	1.51 (4.97E−03)

BIFBRE_03833	NagC-type transcriptional regulator	0.86 (6.02E−01)	1.09 (1.41E−01)	1.06 (3.32E−03)	2.84 (5.26E−03)	2.12 (1.38E−03)	0.97 (1.38E−03)
BIFBRE_03835	Transporter, MFS superfamily	1.24 (3.81E−03)	2.20 (7.00E−01)	0.99 (1.86E−02)	5.13 (4.90E−02)	3.46 (3.33E−02)	1.21 (3.33E−02)
BIFBRE_03836	Sulfatase family protein	1.77 (1.05E−01)	2.47 (1.67E−04)	1.22 (1.86E−02)	2.35 (2.06E−06)	2.21 (1.10E−05)	1.69 (1.10E−05)
BIFBRE_03889	Chorismate synthase	3.24 (3.16E−05)	3.96 (1.51E−05)	2.33 (2.61E−04)	2.59 (1.07E−04)	2.56 (1.59E−04)	3.11 (3.48E−05)
BIFBRE_03895	ATP binding protein of ABC transporter system	2.45 (3.22E−04)	0.83 (6.71E−02)	0.99 (2.72E+04	1.50 (1.64E−03)	0.75 (8.74E−03)	0.83 (8.74E−03)
BIFBRE_03896	ATP binding protein of ABC transporter system	3.09 (5.68E−03)	1.32 (4.81E−01)	1.79 (3.697E−05)	1.54 (1.35E−01)	1.10 (2.96E−01)	1.30 (2.96E−01)
BIFBRE_03897	Oligopeptide transport ATP binding protein	3.33 (5.68E−03)	1.11 (4.81E−01)	1.76 (4.73E−01)	1.19 (1.35E−01)	0.83 (2.96E−01)	1.72 (2.96E−01)
BIFBRE_03898	Cysteine desulfurase/selenocysteine lyase	3.14 (5.68E−03)	1.62 (4.81E−01)	1.72 (3.32E−03)	1.20 (1.35E−01)	0.95 (2.96E−01)	1.84 (2.96E−01)
BIFBRE_03899	IscU protein	2.97 (8.48E−05)	1.09 (1.34E−02)	1.65 (3.32E−03)	1.20 (2.76E−01)	0.95 (7.98E−01)	1.30 (7.98E−01)
BIFBRE_03936	Glycosyltransferase	2.36 (2.21E−05)	2.79 (2.65E−03)	1.61 (3.32E−03)	2.04 (2.45E−05)	2.15 (5.90E−05)	3.37 (5.90E−05)
BIFBRE_04161	*N*-Acylglucosamine 2-epimerase family protein	4.29 (1.49E−05)	2.86 (3.78E−05)	2.57 (3.32E−03)	1.79 (1.51E−05)	2.03 (7.67E−04)	2.52 (7.67E−04)
BIFBRE_04167	Sugar ABC transporter system, permease	4.46 (8.39E−01)	1.25 (8.11E−02)	2.02 (1.86E−02)	1.23 (2.70E−02)	1.42 (8.96E−01)	1.01 (8.96E−01)
BIFBRE_04168	Solute binding protein of ABC transporter system	3.04 (8.39E−01)	1.40 (8.11E−02)	1.94 (1.86E−02)	1.12 (2.70E−02)	1.84 (8.96E−01)	1.14 (8.96E−01)
BIFBRE_04943	Oligopeptide transport ATP binding protein	1.56 (1.74E−04)	2.01 (3.56E−05)	2.08 (4.73E−01)	1.08 (6.76E−05)	0.78 (1.31E−04)	0.77 (1.31E−04)
BIFBRE_04942	Oligopeptide transport system permease protein	2.07 (1.34E−01)	1.80 (2.76E−02)	1.53 (4.73E−01)	0.94 (7.80E−01)	0.76 (3.91E−01)	0.66 (3.91E−01)
BIFBRE_04941	Oligopeptide transport system permease protein	2.73 (1.34E−01)	3.11 (2.76E−02)	1.76 (4.73E−01)	1.19 (7.80E−01)	0.73 (3.91E−01)	0.44 (3.91E−01)
BIFBRE_04896	*N*-Acetylglucosamine-6-phosphate deacetylase	3.08 (3.85E−04)	3.34 (2.35E−03)	3.23 (3.32E−03)	12.62 (2.51E−04)	5.15 (1.08E−04)	12.17 (1.08E−04)
BIFBRE_04895	Glucosamine-6-phosphate isomerase	1.79 (3.85E−04)	3.00 (2.35E−03)	1.74 (3.32E−03)	10.85 (2.51E−04)	3.91 (1.08E−04)	5.57 (1.08E−04)
BIFBRE_04813	Undecaprenol *N*-acetylglucosamine transferase	1.79 (8.63E−02)	2.11 (5.73E−01)	2.37 (1.86E−02)	1.57 (7.52E−01)	1.66 (3.27E−02)	1.43 (3.27E−02)
BIFBRE_04783	Histidinol dehydrogenase	2.72 (4.61E−04)	3.23 (1.86E−04)	2.82 (3.81E−04)	3.27 (1.36E−04)	2.65 (5.86E−04)	2.07 (3.04E−03)
BIFBRE_04540	Galactoside symporter	2.80 (6.21E−04)	2.65 (9.75E−03)	3.23 (3.32E−03)	0.33 (7.04E−04)	0.91 (2.00E−03)	0.41 (2.00E−03)
BIFBRE_04539	β-Galactosidase	2.04 (7.16E−05)	2.02 (1.08E−04)	2.42 (1.91E−04)	0.32 (4.20E−05)	0.32 (5.49E−01)	0.35 (5.49E−01)
BIFBRE_04535	Solute binding protein of ABC transporter system (lactose)	1.73 (6.87E−04)	2.58 (7.97E−04)	0.93 (1.86E−02)	6.83 (2.99E−05)	6.57 (4.79E−05)	6.07 (4.79E−05)
BIFBRE_04494	Permease protein of ABC transporter system	1.99 (9.37E−01)	4.87 (2.67E−02)	1.98 (4.73E−01)	8.35 (4.93E−01)	5.84 (3.48E−01)	4.04 (3.48E−01)
BIFBRE_04493	Solute binding protein of ABC transporter system	2.84 (9.28E−02)	5.61 (2.07E−03)	3.25 (1.86E−02)	13.13 (2.83E−04)	13.13 (1.11E−03)	16.83 (1.11E−03)
BIFBRE_04715	Glutamate transport system permease protein GluC	3.02 (9.37E−01)	3.11 (2.35E−03)	3.51 (3.32E−03)	1.67 (6.76E−05)	1.32 (3.91E−01)	1.15 (3.27E−02)
BIFBRE_04716	Permease protein of ABC transporter	3.22 (3.85E−04)	3.48 (1.86E−04)	3.26 (1.86E−02)	1.35 (2.51E−04)	1.31 (1.08E−04)	0.91 (3.27E−02)
BIFBRE_04187	Hypothetical membrane-spanning protein	1.03 (1.19E−03)	1.54 (9.43E−04)	1.09 (3.32E−03)	4.51 (1.97E−02)	2.52 (3.06E−01)	1.16 (3.06E−01)
BIFBRE_04186	Permease protein of ABC transporter system	1.58 (8.40E−01)	3.04 (1.04E−02)	1.62 (4.73E−01)	8.57 (4.53E−06)	5.22 (1.29E−04)	2.59 (1.29E−04)
BIFBRE_04185	Permease protein of ABC transporter system	0.87 (8.40E−01)	6.19 (1.04E−02)	1.03 (4.73E−01)	30.27 (4.53E−06)	10.63 (1.29E−04)	2.29 (1.29E−04)
BIFBRE_04183	Solute binding protein of ABC transporter system	1.95 (7.68E−01)	8.19 (1.60E−03)	1.73 (1.86E−02	23.85 (2.61E−05)	13.82 (3.37E−04)	8.00 (3.37E−04)
BIFBRE_02812	α-Galactosidase	1.81 (3.32E−03)	2.23 (1.10E−03)	2.12 (2.80E+03)	1.89 (8.67E−04)	4.02 (7.22E−04)	2.01 (7.22E−04)
BIFBRE_04898	Xaa-Pro aminopeptidase	2.81 (3.81E−04)	3.27 (1.19E−03)	3.32 (3.85E−04)	2.61 (2.35E−03)	2.73 (2.35E−03)	3.5 (9.28E−02)
BIFBRE_02830	FimA	2.09 (4.19E−01)	1.82 (2.64E−01)	1.84 (3.32E−03)	2.21 (7.57E−04)	1.80 (8.68E−05)	2.19 (8.68E−05)
BIFBRE_02843	ATP binding protein of ABC transporter system	4.65 (3.54E−02)	4.01 (1.71E−01)	4.21 (4.50E+04)	11.84 (6.80E−02)	6.41 (5.67E−01)	6.41 (5.67E−01)
BIFBRE_02846	PTS, IIC component	2.35 (3.54E−02)	3.13 (1.71E−01)	2.35 (4.73E−01)	2.11 (6.80E−02)	3.47 (5.67E−01)	3.24 (5.67E−01)

[a] Values are the changes for each sample in comparison with the reference sample. The *P* values were calculated from replicate measurements of two different hybridization experiments.

complete putative ABC peptide transporter system, including the solute binding subunit (BIFBRE_03436), the permease (BIFBRE_03444), and the ATP binding protein (BIFBRE_02843), as well as a PTS (BIFBRE_02846).

Additional genes that were found to be upregulated regardless of the specific milk substrate utilized for *B. breve* 4L cultivation included the *tadE*/*F* genes (BIFBRE_03022 and BIFBRE_03023), which are part of the *tad* locus. In *B. breve* UCC2003, the products of the *tad* genes have been reported to be responsible for gut colonization and for the persistence of this commensal in mouse intestine (18). It is thus interesting that dietary components such as animal- or plant-derived milks appear to induce transcription of genes whose products mediate interaction of bifidobacterial cells with their hosts. Such results suggest an interesting level of coevolution between vegetables used for human consumption and their effect on their human host, based on selection of intestinal bifidobacteria.

Conclusions. In recent times, scientific data have emerged on how specific bifidobacterial species colonize the infant gut (4, 14), although very little is currently known about the contribution of an infant's nourishment to the overall microbial population structure. It has been proposed that specific components of human milk, i.e., HMOs, have a significant impact on the composition of the intestinal microbiota of infants and represent a fascinating example of reciprocal evolution between mammals and their inhabiting commensals (22, 23). The identification of *B. breve* as the predominant bifidobacteria taxon present in human milk corroborates previously published data (10, 19) and reinforces the hypothesis that members of this species are important colonizers of the infant gut.

The genomic knowledge on how growth of bifidobacteria can be sustained in milk is important for understanding the molecular mechanisms governing the initial stages of bacterial colonization of the human gut. Such knowledge is of pivotal importance for gaining insights into host-microbe interactions and the produced beneficial effects of the bifidobacterial population. It is worth mentioning that the molecular dissection of the metabolic capabilities of *B. breve* 4L described here is based on a reductionist vision that does not consider the concomitant coexistence of different strains/species, which is a common feature of the human gut. In fact, the breakdown of complex substrates, such as those present in milk in the human gut, is the consequence of the collective action of the enzymes produced by the various members of the microbiota. Furthermore, the identification of genes expressed by *B. breve* might be important as potentially catabolizing components necessary for the degradation of human milk. Future research activities will be carried out in this direction in order to understand how different members of the gut microbiota may influence each other, and the use of the infant gut microbiota may prove an appropriate model in this respect since, in contrast to the adult type, the infant intestinal microbiota is rather simple.

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